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Exploring a neurogenic basis of velopharyngeal dysfunction in *Tbx*1 mutant mice: No difference in volumes of the nucleus ambiguus☆

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ABSTRACT

Objective: Velopharyngeal hypotonia seems to be an important factor in velopharyngeal dysfunction in 22q11.2 deletion syndrome, but the etiology is not understood. Because *TBX*1 maps within the typical 22q11.2 deletion and *Tbx*1-deficient mice phenocopy many findings in patients with the 22q11.2 deletion syndrome, *TBX*1 is considered the major candidate gene in the etiology of these defects. *Tbx*1 heterozygosity in mice results in abnormal vocalization 7 days postnatally, suggestive of velopharyngeal dysfunction. Previous case–control studies on muscle specimens from patients and mice revealed no evidence for a myogenic cause of velopharyngeal dysfunction. Velopharyngeal muscles are innervated by cranial nerves that receive signals from the nucleus ambiguus in the brainstem. In this study, a possible neurogenic cause underlying velopharyngeal dysfunction in *Tbx*1 heterozygous mice was explored by determining the size of the nucleus ambiguus in *Tbx*1 heterozygous and wild type mice.
Methods: The cranial motor nuclei in the brainstems of postnatal day 7 wild type ($n = 4$) and *Tbx*1 heterozygous ($n = 4$) mice were visualized by *in situ* hybridization on transverse sections to detect *Islet*-1 mRNA, a transcription factor known to be expressed in motor neurons. The volumes of the nucleus ambiguus were calculated.
Results: No substantial histological differences were noted between the nucleus ambiguus of the two groups. *Tbx*1 mutant mice had mean nucleus ambiguus volumes of 4.6 million μm^3 (standard error of the mean 0.9 million μm^3) and wild type mice had mean volumes of 3.4 million μm^3 (standard error of the mean 0.6 million μm^3). Neither the difference nor the variance between the means were statistically significant (*t*-test $p = 0.30$, Levene's test $p = 0.47$, respectively).
Conclusions: Based on the histology, there is no difference or variability between the volumes of the nucleus ambiguus of *Tbx*1 heterozygous and wild type mice. The etiology of velopharyngeal hypotonia and variable speech in children with 22q11.2 deletion syndrome warrants further investigation.

1. Introduction

The 22q11.2 deletion syndrome (22q11DS) is the most frequent survivable human syndrome that is caused by a hemizygous microdeletion within a chromosome [1]. In approximately 85% of all 22q11DS patients, a 3 megabase (Mb) region on chromosome 22 is deleted [2] containing about 45 genes [3]. One of the genes that maps within the deleted region is *Tbx*1, which is expressed in pharyngeal endodermal pouches, in pharyngeal mesoderm including the mesodermal cores of the pharyngeal arches, and in head mesenchyme during embryonic development [1] and in the brain after birth [4]. Major phenotypes of 22q11DS can be related to aberrant development of the pharyngeal arches and pouches 3, 4, and 6, including facial dysmorphism, feeding and speech problems due to velopharyngeal dysfunction (VPD), hypocalcaemia due to parathyroid dysfunction, immune disorders due to thymus dysfunction, and congenital heart disease.

VPD occurs when the valve mechanism of the soft palate and the lateral and posterior pharyngeal walls fail to close the port between the oral and nasal cavities, resulting in hypernasal speech. Some children with VPD undergo surgery to decrease the size of the velopharyngeal port. In general, postoperative residual VPD is more prevalent among children with 22q11DS than in children without the syndrome [5–11], but some patients with 22q11DS fare as well as their non-syndromic counterparts [12–17]. It is not clear why some children with 22q11DS benefit more from surgery than others [7,18]. Phenotype variability of VPD in 22q11DS has been one of the research foci of the 22q11DS team at our tertiary hospital.

All surgical techniques rely on some intrinsic muscle activity for closure of the remaining velopharyngeal port [19]. A possible

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explanation for the different postoperative outcomes is a neuromuscular component of VPD in 22q11DS as seen on nasendoscopic views of attempted velopharyngeal closure [20]. On magnetic resonance imaging, the pharyngeal constrictor muscle in patients with 22q11DS was found to be hypotrophic compared to controls [21], which may be the result of abnormal development of the muscle or its innervation. The etiology of velopharyngeal hypotonia is uncertain, but may primarily result from myogenic or neurogenic abnormalities. Superior constrictor muscle biopsies taken from children with and without 22q11DS revealed no clear histological differences, suggesting a nonmyogenic origin of velopharyngeal hypotonia in patients with 22q11DS [22]. Whether a neurogenic cause underlies VPD in patients with 22q11DS is unclear.

Neurogenic pharyngeal weakness is seen in amyotrophic lateral sclerosis, a neurodegenerative disease accompanied by a decreased number of cells in the brainstem nucleus ambiguus (nA) [23,24]. The nA transmits signals from the cerebral cortex to the vagal (n.X) and accessory (n.XI) cranial nerves which innervate the pharyngeal muscles [25–27]. Additionally, some patients with Möbius syndrome, which is characterized by congenital weakness or paralysis of the muscles innervated by the facial nerve (n.VII), have hypoplastic brainstem facial cranial nerve nuclei with fewer neurons than controls [28–30]. Similarly, congenital VPD in 22q11DS could be caused by hypoplastic development of the nA. Unfortunately, noninvasive imaging does not permit an accurate estimation of the size the brainstem nuclei [31], necessitating a histological analysis of brainstem tissue.

Postmortem human brainstem material is difficult to obtain, therefore we resorted to studying an animal model of 22q11DS. Among vertebrate model organisms, the neuronal architecture of the mouse is the most similar to that of humans [32]. Mouse models for 22q11DS have been generated by deleting a 1 Mb homologous region on mouse chromosome 16 (*Df*(16)1, *LgDel*) including *Tbx*1, or specifically disrupting the *Tbx*1 gene [33,34]. The phenotype of *Tbx*1 heterozygous mutant mice ($Tbx1^{+/-}$) is less penetrant and does not phenocopy the entire phenotypic spectrum of patients with 22q11DS. However, recent findings demonstrated that seven to eight-day-old $Tbx1^{+/-}$ mouse pups (P7–8) may have VPD since they vocalize at a lower frequency and for a shorter duration compared to wild type littermates [35]. Interestingly, a loss-of-function point-mutation of *TBX*1 in patients without the typical 22q11.2 deletion, results in phenotypes similar to those found in patients with 22q11DS, including VPD [36]. Therefore, $Tbx1^{+/-}$ mice can be used as an adequate model to study the VPD phenotype found in 22q11DS.

Moreover, as in patients with 22q11DS, phenotypic variance is seen in the $Tbx1^{+/-}$ mouse model [37]: all $Tbx1^{+/-}$ embryos have fourth pharyngeal arch artery hypoplasia at E10.5, but at term only 30–50% have fourth pharyngeal arch artery-derived cardiovascular defects [33]. The differences in phenotypic penetrance depends on the genetic background of the mouse strains [37–39], and on genetic modifiers including *Vegfa*, *Nrp*1, *Spry*, and retinoic acid [40–44].

The presence of velopharyngeal hypotonia as underlying cause for the VPD was not specifically mentioned in the study with mouse pups [35] nor in the study with patients with the *TBX*1 point-mutation [36]. The requirement of *Tbx*1 during development of velopharyngeal muscles and nerves has been shown in *Tbx*1-deficient ($Tbx1^{-/-}$) mice which die during fetal and neonatal stages: $Tbx1^{-/-}$ mice have hypoplastic branchiomeric head and neck muscles [45,46] and abnormally fused ganglia of the glossopharyngeal (n.IX) and n.X nerves [47,48]. Thus, although *Tbx*1 is not expressed in primary neural crest cells [49], the neural crest-derived ganglia are aberrantly formed in the absence of *Tbx*1 [50].

The objective of this study was to explore the possibility that a neurogenic defect causes velopharyngeal hypotonia in 22q11DS by comparing the gross histology of the nA in the $Tbx1^{+/-}$ mouse model for 22q11DS to that of wild type mice. Diminished or absent activity of *Tbx*1 gene may indirectly effect the brainstem as it does the cranial nerves [47,48]. Our results indicate that the volume of the nA is not significantly affected by *Tbx*1 haplosufficiency.

2. Materials and methods

2.1. Mice

$Tbx1^{+/lacZ}$ mice [33] were intercrossed to generate wild type and heterozygous mutant pups. Genotypes were confirmed by PCR using primers specific for the *lacZ* gene [33]. All mice were maintained on an FVB background. Animal care was in accordance with national and institutional guidelines. The experimental procedure was approved by the animal ethics committee of the Academic Medical Center in Amsterdam, the Netherlands. On postnatal day 7 (P7) the pups ($n = 4$ of each genotype) were brought into a hypercapnic coma in a sealed cage and sacrificed for tissue isolation. The brainstems were isolated in ice-cold phosphate-buffered saline (PBS 1x), fixed by overnight immersion in 4% paraformaldehyde (PFA), and embedded in paraplast for further processing.

2.2. In situ hybridization

Embedded brainstem tissue was cut into 10 μm thick transverse sections with a Leica RM 2165 rotation microtome, mounted on Starfrost slides, and processed for non-radioactive *in situ* hybridization (ISH) as described [51]. The brainstem motor nuclei were visualized by ISH with a DIG-labeled *Islet*-1 (*Isl*1) [52,53] mRNA probe [51]. The sections were photographed using a camera connected to a Zeiss Axiophot microscope.

2.3. Outcome

Morphometric analyses were performed blinded using imaging software (Amira 5.4, Visage Imaging, San Diego, CA, USA). The nA of the mutant and the wild type pups were compared qualitatively by describing the appearance, and quantitatively by calculating the volume marked by *Isl*1. Rather than measuring every section that contained the nA, the surface area of the nA on a minimum of 10 equally spaced sections encompassing the nA were measured. Using Cavalieri's principle, the sum of the measured areas was multiplied by the distance between the selected sections (Fig. 1). This approximation of the volume is accurate to within 5% of the true volume [54]. The volumes of the nA of $Tbx1^{+/-}$ and wild type mice were compared using a two-tailed *t*-test. The variance was measured with Levene's test. Statistical calculations were performed using IBM SPSS Statistics for Windows (Version 20.0. Armonk, NY, USA).

2.4. Sample size calculation

The number of pups needed to obtain statistically significant results, was determined based on a study in which n.X innervation of the stomach was compared between wild type and $Tbx1^{+/-}$ mice [48]. At embryonic day 16.5 (E16.5), significantly less n.X fibers intersected in the stomachs of $Tbx1^{+/-}$ mice ($n = 9$) than in wild type mice ($n = 9$) (14.6 ± 1.6 *vs.* 20.4 ± 1.3, $p < 0.05$). With these numbers, the required sample size to find a similarly significant difference in nA volumes between the genotypes, with an alpha of 0.05, and a power of 0.80 is only $n = 2$ pups per genotype. Since this calculation is based on the n.X and not the nA, this number was

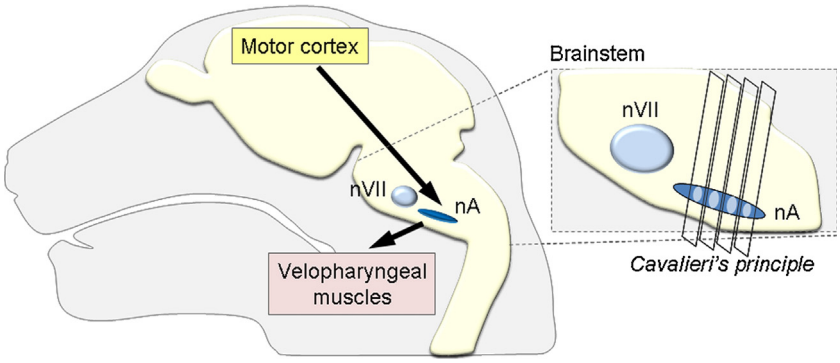

Fig. 1. Methods. Sagittal view of a mouse brain and brainstem showing the locations of the facial nucleus (nVII) and nucleus ambiguus (nA). Signals from the cerebral motor cortex are relayed to the velopharyngeal muscles *via* the nA. Inset showing magnification and Cavalieri's principle of calculating the volume of a structure based on equally spaced transverse sections.

empirically doubled so $n = 4$ pups per genotype were used in this pilot study.

3. Results

To test whether a neurogenic abnormality underlies velopharyngeal hypotonia in patients with 22q11DS, we harvested and analyzed brainstems from *Tbx*1$^{+/-}$ ($n = 4$) and wild type ($n = 4$) pups from two litters. No macroscopic qualitative differences were noted between both genotypes. As previously described [55–57], the spindle-shaped nA extends cranio-caudally from the facial nucleus (nVII) to the pyramidal decussation [58]. To identify and localize the nA (Fig. 2A), we used a riboprobe directed against *Isl*1 mRNA, encoding a LIM domain-containing transcription factor. *Isl*1 is expressed in motor neurons of all cranial nerves, including the oculomotor, trochlear, trigeminal, abducens, facial, ambiguus, and hypoglossal nuclei in the brainstem [52,59,60]. This approach furthermore allowed the identification of the facial nucleus directly cranial to the nA and the hypoglossal nucleus dorsal to the nA [61], facilitating the localization of the nA. We found that within the nA, the cranially located neurons are packed more compactly and the caudally located neurons are more loosely arranged (Fig. 2B). The nA in the two genotype groups did not differ in shape or cell density. Quantitatively, *Tbx*1$^{+/-}$ mutant pups had mean nucleus ambiguus volumes of 4.6 million μm^3 (standard error of the mean (SEM) 0.9 million μm^3) and wild type mice had mean volumes of 3.4 million μm^3 (SEM 0.6 million μm^3) (Fig. 2C). The difference between the means was not statistically significant (*t*-test $p = 0.30$), nor was the variance (Levene's test $p = 0.47$).

4. Discussion

Velopharyngeal hypotonia is a common cause of VPD in patients with 22q11DS. However, the etiology and subsequent speech problems in children with 22q11DS is still poorly understood. We previously demonstrated that myogenic disturbances did not seem to underlie VPD in 22q11DS children [22]. In this study, we aimed to determine whether a neurogenic cause underlies velopharyngeal hypotonia. The size of the nA is decreased in other diseases with velopharyngeal hypotonia [23,24,28–30]. We measured the volume of the nA in mice heterozygous for *Tbx*1, the major candidate gene in the etiology of 22q11DS, and did not observe a clear difference or variability in the volumes of the nA compared with wild type mice.

4.1. Evidence of neurologic deficits in Tbx1 mouse mutants and 22q11DS human patients

The lack of difference in nA volumes between the genotypes does not disprove a neurologic etiology of the supposed VPD in *Tbx*1$^{+/-}$ mice. Cerebral deficits are apparent in *Tbx*1$^{+/-}$ mice: relative to wild type mice, they have reduced prepulse inhibition [4], lower grip strength, and delayed movement initiation [62]. The defects seem to be subtle: even in the *LgDel* model, adult mouse brains show no significant changes in weight or gross morphological appearance [63]. *Tbx*1 expression is limited to the brain vasculature, suggesting that microvascular abnormalities contribute to the phenotypes found in these mutants [4]. Distal to the nA, the morphology and volumes of the n.X ganglia do not differ between *Tbx*1$^{+/-}$ and wild type mouse embryos, but a significant

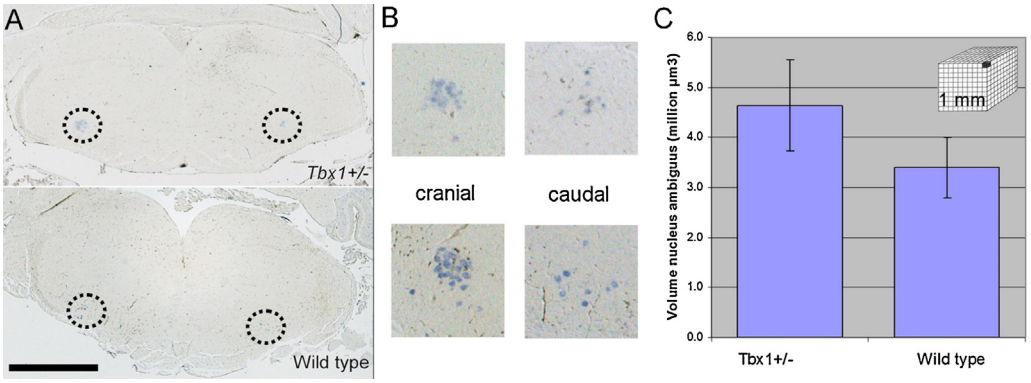

Fig. 2. Nucleus ambiguus of *Tbx*1$^{+/-}$ and wild type P7 pups. (A) *Isl1 in situ* hybridization-labeled transverse brainstem sections. Scale: bar = 1 mm. (B) Magnification of nucleus ambiguus, showing cranial compact and caudal loosely spread cells. (C) Mean volumes of the nucleus ambiguus. Error bars = standard error of the mean. Scale: small dark cube = 1 million μm^3.

decrease in the number of n.X fibers that intersect the stomach was observed in *Tbx*1$^{+/-}$ mutants compared to wild type mouse embryos, suggesting defective n.X projections [48]. Pharyngeal projections have not been studied beyond embryonic day 10 thus far [47].

Clinically, impaired n.X function as evidenced by velar paresis (n = 10/13) [64], as well as velar and pharyngeal motion are negatively affected in 22q11DS patients compared to nonsyndromic patients with a repaired cleft palate [65,66]. Moreover, dysfunction of the muscles normally innervated by the nA in 22q11DS are suggested to cause polyhydramnios [67,68] due to swallowing disorders [69], a hoarse voice [5,70–75] presumably due to laryngeal muscle hypotonia [76,77], and case reports of aspiration [68,78,79]. These phenotypic characteristics could result from neurogenic or myogenic abnormalities.

The neurogenic component of the syndrome needs attention. In some patients with 22q11DS the velopharyngeal valve mechanism is sufficient during swallowing, but not during speech. The nA relays motor signals to the velopharynx as well as to the intrinsic muscles of the larynx and the upper esophagus. The nA is active during vocalization, respiration, sneezing, coughing, swallowing, and the gag reflex [25]. In all motor neurons that are related to both swallowing and vocalization, higher electromyographic (EMG) activity levels were achieved during swallowing, reflecting that more forceful adduction of the vocal folds is needed for glottal closure during swallowing to protect the airway against aspiration [80]. If the muscles can be sufficiently forcefully activated during reflexive swallowing in 22q11DS, hypotonia during speech may indicate impairment of the cerebral voluntary component of vocalization.

4.2. Limitations of this study

The *Tbx*1 mouse background used in this study (FVB) differs from that used in the vocalization study (C57BL/6J) [35], which could affect the penetrance of defects [37,38], because an FVB genetic background offers a protective effect with regard to fourth pharyngeal arch artery development [39]. Therefore, an FVB background could also be protective with regard to nA development and VPD. Additionally, the phenotypic variability of 22q11DS may reflect the array of genetic variability in humans which could not be recapitulated in experimental animals with a homogeneous background [45].

Another important limitation of this study is that only the histology of the nA has been studied by labeling *Isl*1. No evidence has been presented that could prove normal function. During embryology and postnatal growth, morphology and physiological function are not necessarily temporally coincident [81]; abnormalities may be apparent on electrophysiological examination without histopathological abnormalities [32].

Finally, in 22q11DS typically around 45 genes are deleted. Although the lower frequency and decreased duration of vocalization in *Tbx*1$^{+/-}$ mutants suggests that this gene may primarily contribute to VPD, other genes could function in parallel or in the same genetic pathway [46]. Other candidate genes in the deleted region include CLTD which is expressed in skeletal muscle, its deletion may contribute to hypotonia [82]; CRKL which is expressed in migrating neural crest cells; its deletion results in hypoplastic n.IX and n.X [83]; and Cdcrel-1(Pnut1) which is expressed in the n.IX and n.X [84]. Haplosufficiency of six other genes (*Slc*25*a*1, *Prodh*, *Mrpl*40, *αZdhhc*8, *Txnrd*2, and *T*10) deleted in the syndrome might negatively affect synaptogenesis which peaks at P0 [63]. Haplosufficiency of three other genes (DGCR6(L) and PRODH) may contribute to neurochemical imbalance in the excitatory and inhibitory neurotransmitters GABA and glutamate [85,86].

4.3. Future studies

To further differentiate between a neurogenic and myogenic etiology of velopharyngeal hypotonia in 22q11DS, an invasive neuromuscular conduction study (EMG) of the velopharyngeal muscles could be performed [87]. Electrophysiological analysis in distinct regions of the brain in wild type and *Tbx*1 heterozygous mice should shed light on the contribution of the cerebral cortex to velopharyngeal closure [63].

5. Conclusions

This study is a step in the process of unraveling the hitherto inadequately explained variation between genotype and phenotype in 22q11DS [37]. The phenotypes among patients with 22q11DS vary greatly [3,37]. Parents and caretakers of children with 22q11DS are left with many questions about the likely manifestations and the course of these problems in time. Mice deficient for *Tbx*1 are known to display a variety of abnormalities similar to those described in 22q11DS, but the mutation does not seem to affect the morphology or volume of the nA since there were no differences between *Tbx*1 heterozygous and wild type mice. The volumes did not vary more within the mutant group than in the wild type group. The underlying cause of velopharyngeal hypotonia and subsequent speech problems in children with 22q11DS continues to be unknown. It is likely that genetic modifiers beyond the 22q11DS genes play a key role in determining 22q11DS phenotypic severity [63]. With increasing knowledge, the parents and caretakers of children with 22q11DS can be better informed about the expected outcome after velopharyngeal surgery for VPD. Finally, improving our understanding the underlying mechanisms that cause VPD in 22q11DS may lead to novel therapeutic and/or diagnostic methods.

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